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Cloning the P. falciparum Gene Encoding PfEMP1, a Malarial Variant Antigen and Adherence Receptor on the Surface of Parasitized Human Erythrocytes

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Summary

Plasmodium falciparum-infected human erythrocytes evade host immunity by expression of a cell-surface variant antigen and receptors for adherence to endothelial cells. These properties have been ascribed to P. falciparum erythrocyte membrane protein 1 (PfEMP1), an antigenically diverse malarial protein of 200–350 kDa on the surface of parasitized erythrocytes (PEs). We describe the cloning of two related PfEMP1 genes from the Malayan Camp (MC) parasite strain. Antibodies generated against recombinant protein fragments of the genes were specific for MC strain PfEMP1 protein. These antibodies reacted only with the surface of MC strain PEs and blocked adherence of these cells to CD36 but without effect on adherence to thrombospondin. Multiple forms of the PfEMP1 gene are apparent in MC parasites. The molecular basis for antigenic variation in malaria and adherence of infected erythrocytes to host cells can now be pursued.

Introduction

Erythrocytes infected with the malaria parasite Plasmodium falciparum disappear from the peripheral circulation as they mature from the ring stage to trophozoites (Bignami and Bastianeli, 1889). This phenomenon, known as sequestration, results from parasitized erythrocyte (PE) adherence to microvascular endothelial cells in diverse organs (Miller, 1969). Sequestration is associated temporally with expression of knob protrusions (Leech et al., 1984a), expression of a very large antigenically variant surface protein, called P. falciparum erythrocyte membrane protein 1 (PfEMP1) (Aley et al., 1984; Leech et al., 1984b; Howard et al., 1988), and expression of new receptor properties that mediate adherence to endothelial cells (Miller, 1969; Udeinya et al., 1981). Endothelial cell surface proteins such as CD36, thrombospondin (TSP), and intercellular adhesion molecule 1 (ICAM-1) (Pasloske and Howard, 1994) have been identified as major host receptors for mature PEs.

PE sequestration confers unique advantages for P. falciparum parasites (Howard and Gilladoga, 1989), but also contributes directly to the acute pathology of P. falciparum (Miller et al., 1994). Adherence of PEs to cerebral blood vessels and consequent local microvascular occlusion is clearly a major contributing factor (Howard and Gilladoga, 1989; Patnaik et al., 1994).

The capacity of P. falciparum PEs to express variant forms of PfEMP1 contributes to the special virulence of this parasite. Variant parasites can evade variant-specific antibodies elicited by earlier infections. The P. falciparum variant antigens have been defined in vitro by use of antiserum prepared in Aotus trivergatus monkeys infected with individual parasite strains (Howard et al., 1988). Antibodies raised against a particular parasite will only react by PE agglutination, indirect immunofluorescence, or immunoelectron microscopy with PEs from the same strain (van Schravendijk et al., 1991). Such studies with PEs from malaria patients in diverse geographic locations and sera from the same or different patients amply confirm that PEs in natural isolates express variant surface antigens and that individual patients respond to infection by production of isolate-specific antibodies (Marsh and Howard, 1986; Aguiar et al., 1992). Expression of a variant antigen on PEs has also been demonstrated in several simian, murine, and human malaria species, including P. knowlesi (Brown and Brown, 1965; Barnwell et al., 1983), P. chabaudi (Gilks et al., 1990; Brannan et al., 1994), P. fragile (Handunnetti et al., 1987), and P. vivax (Mendis et al., 1988). Laboratory studies with P. knowlesi (Brown and Brown, 1965; Barnwell et al., 1983) or P. falciparum (Hommel et al., 1983) in monkeys and P. chabaudi in mice (Gilks et al., 1990) confirmed that antigenic variation at the PE surface is associated with prolonged or chronic infection and the capacity to reestablish blood infection repeatedly in previously infected animals. Studies with cloned parasites demonstrated that antigenic variants can arise with extraordinary frequency, 2% per generation with P. falciparum (Roberts et al., 1992) and 1.6% per generation with P. chabaudi (Brannan et al., 1994).

PfEMP1 was identified as a size-diverse (200–350 kDa) ^{125}I-labeled protein on PEs that is lacking from uninfected erythrocytes and can also be labeled by biosynthetic incorporation of radiolabeled amino acids (Leech et al., 1984b; Howard et al., 1988). PfEMP1 is not extracted from PEs by neutral detergents such as Triton X-100 but is extracted by sodium dodecyl sulfate (SDS), suggesting linkage to the erythrocyte cytoskeleton (Aley et al., 1984). After addition of excess Triton X-100, PfEMP1 is immunoreactive with appropriate serum antibodies (Howard et al., 1988). Mild trypsinization of intact PEs rapidly cleaves PfEMP1 from the cell surface (Leech et al., 1984b). PfEMP1 bears antigenically diverse epitop s, since it is immunoprecipitated from particular strains of P. falciparum by antibodies from sera of Aotus monkeys infected with th sam strain, but not by antibodies from animals inf cted with heterologous strains (Howard et al., 1988). Knobless PEs derived from parasite passage in splen ctomized Aotus monkeys (Aley et al., 1984) do not express surface PfEMP1 and ar not agglutinated with s ra from immun individuals

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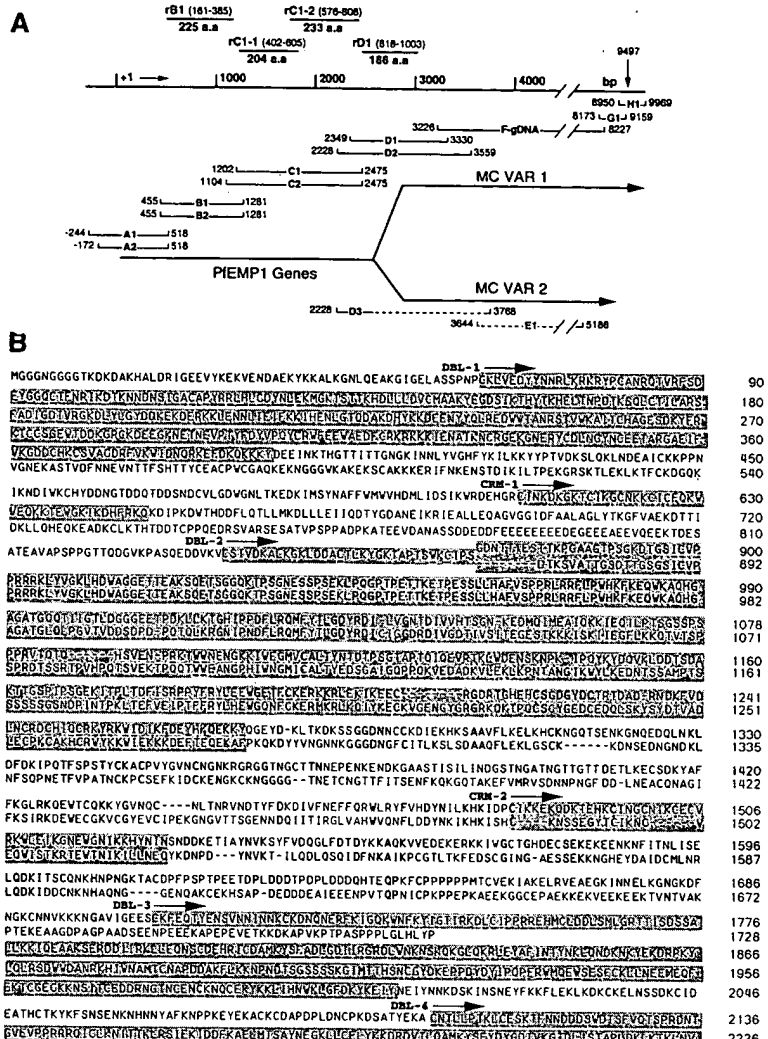

Figure 1. Map of cDNA and gDNA Clones and Deduced Amino Acid Sequence of *MCvar-1* and *MCvar-2* PfEMP1 Genes of K⁺ MC Parasites

(A) Map of the two MC PfEMP1 genes and recombinant protein fragments. The predicted open reading frame is shown starting from nucleotide +1 until the stop codon at position 9497 of *MCvar-1* indicated by a vertical arrow. DNA clones are located with their boundaries (nucleotide number). Clones A1 via D2 and F-gDNA to clone H1 describe the *MCvar-1* gene. Clones A1 via D3 to E1 describe the *MCvar-2* gene. Each clone was shown to be physically linked to the adjacent clones, thus confirming *MCvar-1* and *MCvar-2* as two independent PfEMP1 genes. The regions of PfEMP1 expressed as GST fusion proteins, rB1, rC1-1, rC1-2, and rD1, are shown, with the total amino acid length of each and boundary amino acids.

(B) The predicted amino acid sequence of the two MC PfEMP1 genes deduced from cDNA and gDNA clones. The position of the putative 725 bp intron of *MCvar-1* (nucleotides 7429–8153) is indicated by a vertical arrow. The likely membrane-spanning domain is boxed. Both genes have identical sequence until amino acid 871. The extensive sequence differences of *MCvar-1* and *MCvar-2* are shown as separate sequences until the end of the known sequence of *MCvar-2*. Where the sequence diverges, *MCvar-1* is written above *MCvar-2*. Amino acid sequence identity in these two sequences is shown in boldface. The four DBL domains and the three CRMs are shaded. Consensus amino acids in each DBL domain are underlined, and conserved cysteines of the CRM motif are indicated by underlined dots.

or infected monkeys (Howard et al., 1988; Howard and Gilladoga, 1989). In general, sera that react with the PE surface by indirect immunofluorescence and antibody-mediated PE agglutination are the only sera to immunoprecipitate ^{125}I-labeled PfEMP1 from any particular strain (Howard et al., 1988; van Schravendijk et al., 1991; Biggs et al., 1992).

Several pieces of indirect evidence have linked expression of PfEMP1 with the acquisition of new host protein r c ptor properties on the surface of PEs (Howard and Gilladoga, 1989; Pasloske and Howard, 1994). Recently we d monstrat d that solubilized PfEMP1 can bind to CD36, TSP, or ICAM-1 and that tryptic fragments of PfEMP1 cleaved from th PE surface bind to TSP or CD36 (Baruch et al., unpublished data). Hence, PfEMP1 is responsible for both antigenic variation and receptor properties on PEs, both central to the special virulence and pathology of P. falciparum.

In this report, we use these properties to demonstrate that novel P. falciparum cDNAs encode the PfEMP1 protein of Malayan Camp (MC) strain K⁺ (for knob-positive) parasites. The role of PfEMP1 in antigenic variation and adherence of PEs to host proteins can now be explored at the molecular level.

R sults

Id ntification f a N v l cDNA

Earlier w described the preparation of a rabbit antiserum, 05-75, that reacted with two very large (>300 kDa) malarial proteins, PfEMP1 and PfEMP3, associated with the erythrocyte m mbrane of P. falciparum PEs (van Schravendijk

et al., 1993). Adsorption of antiserum 05-75 against a recombinant protein corresponding to part of PfEMP3 (β-gal-12.1.3) ablated immunoreactivity with PfEMP3 without effect on immunoprecipitation of ^{125}I–PfEMP1 (van Schravendijk et al., 1993). Screening of a genomic DNA (gDNA) λgt11 expression library from MC parasites with 05-75 antiserum preadsorbed with the λ12.1.3 clone identified one insert of 1.8 kb, denoted A6-2, that hybridized to a large (8–10 kb) mRNA band (data not shown). Recombinant protein derived from part of clone A6-2 blocked immunoprecipitation of MC PfEMP1 by antiserum 05-75 (data not shown). In view of these results, we continued to explore this gDNA clone. A cDNA library of MC K$^+$ strain parasites was subjected to polymerase chain reaction (PCR) with primers derived from A6-2 to generate cDNA clones extending 5′. One of these clones, of 1.1 kb denoted clone 2-3-2, proved to be highly unstable in Escherichia coli. Nevertheless, with repeated PCR using diverse probes from clone 2-3-2, we identified a more stable cDNA, denoted D1 (Figure 1A), which was identical at its 3′ end to part of clone 2-3-2. Additional contiguous cDNA or gDNA clones extending 5′ and 3′ from clone D1 were produced (Figure 1A). Small differences in nucleotide sequence and deduced amino acid sequence were observed in several of the overlapping cDNA clones. However, none of the clones had sequence overlapping with clone A6-2. Thus, clone 2-3-2 probably resulted from recombination where clone A6-2 may represent the 3′ end of a PfEMP1 gene variant from the one described here. PCR products of the expected size were generated from MC K$^+$ gDNA and cDNA with several sets of primers, followed by oligonucleotide hybridization and direct sequencing to verify that the clones shown in Figure 1A are contiguous.

Structure of the Gene and Variant Sequences

Two members of the PfEMP1 gene family that have almost identical sequence at their 5′ ends (clones A–C) were cloned (Figure 1A). Clone A1 included 244 nt before a start codon and initiation of an open reading frame that extended through overlapping cDNA clones via D3 to clone E1 (5186 nt, *MCvar-2*; Figure 1A), or to a stop codon at position 9497 in clone H1 via D2 and the F-gDNA clone (9969 nt, *MCvar-1*; Figure 1A). We have generated PCR products from gDNA and cDNA that span clones B1 to F-gDNA (1120–3702, 2582 bp) and clones A1 to D1 and D2 (1–3009, 3009 bp), as well as PCR products from gDNA and cDNA that span clones B1 to E1 (1204–4139, 2935 bp) and B1 to D3 (1120–3612, 2492 bp). Hence, *MCvar-1* and *MCvar-2* are two independent PfEMP1 genes independently expressed in MC strain parasites. The genomic location of *MCvar-1* and *MCvar-2* is not yet known. These genes have overlapping sequence with a single amino acid substitution between cDNA clones D2 and D3 up to amino acid 871, at which point their sequences diverge (Figure 1B). A 725 bp intron was identified in the *MCvar-1* gene at nucleotide 7429 by comparison with sequenc data obtained from other genes of the family described in an accompanying report (Su et al., 1995 [this issue of *Cell*]). The *MCvar-1* sequence is full length, while the sequence of *MCvar-2* represents most of the extracellular domain of PfEMP1. The deduced amino acid sequences of *MCvar-1* (2924 amino acids) and *MCvar-2* (1729 amino acids) are shown in Figure 1B. Small differences in nucleotide sequence and deduced amino acid sequence were observed in several of the overlapping cDNA clones (data not shown).

The amino acid sequence from MC strain parasites shows extensive homology with the several sequences in a gene family identified in the companion report from Su et al. (1995). *MCvar-1* has four domains homologous with the Duffy binding–like (DBL) domains of P. vivax, P. falciparum, and P. knowlesi, each containing five consensus motifs rich in cysteine residues (Figure 1B; Su et al., 1995). Three cysteine-rich motifs, denoted CRM-1–CRM-3, wer found outside the DBL domains located after DBL-1, DBL-2, and DBL-4, respectively (Figure 1B). Analysis of the SwissProt, NBRF-PIR, and translated GenBank (rel ase 86) databases identifies the sequences as novel.

An RGD motif (amino acids 1212–1214) and an LDV motif (amino acids 142–144) that could be involved in protein–protein adhesion (Kuhn and Eble, 1994) are evident in the sequence (Figure 1B). The deduced amino acid sequence lacked stretches of tandem repeats. A single transmembrane domain (*MCvar-1*, amino acids 2450–2475) was identified, followed by a presumed intracellular domain encoded by the 3′ exon. The majority of the protein sequence is therefore extracellular. This is consistent with the combined size of tryptic fragments of MC PfEMP1 (210 kDa) generated from trypsin treatment of intact PEs (Baruch et al., unpublished data).

Southern blotting of fragments of the novel genes with EcoRI- or EcoRI–HindIII-digested gDNA was performed

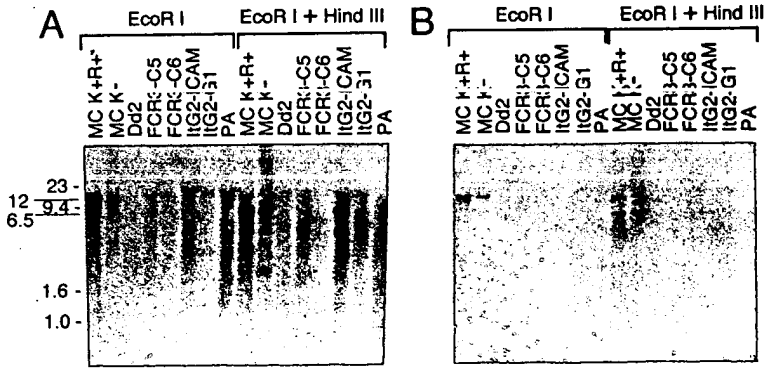

Figure 2. Southern Blots Reveal Shared and Strain-Specific Portions of the PfEMP1 Gene in Different Parasites

Autoradiographs of Southern blot hybridization of cDNA clones from the MC PfEMP1 genes with DNA from various P. falciparum parasites digested with EcoRI or EcoRI and HindIII.
(A) Probing with cDNA A1, from the 5′ end of the gene, shows hybridization to multiple bands with all P. falciparum parasites tested.
(B) Probing with clone C1 shows hybridization to fewer bands with MC K$^+$ and MC K$^-$ parasites only. Markers of molecular mass, in kilobases, are indicated on the left.

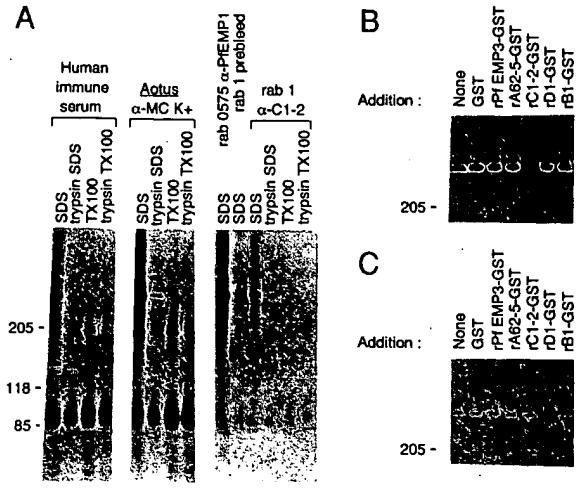

Figure 3. Immunization with Recombinant Proteins Elicits Noncross-reactive Antibodies That Immunoprecipitate ^{125}I–PfEMP1 from MC K$^+$ PEs

(A) The ^{125}I-labeled band immunoprecipitated by anti-rC1-2 antisera shares properties with PfEMP1. MC K$^+$ PEs were radioiodinated and some of the cells treated with trypsin (5 min, 10 μg/ml). Sequential Triton X-100 and SDS extracts were immunoprecipitated with three antisera that define ^{125}I–PfEMP1 of MC K$^+$ parasites: a pool of human immune serum; Aotus anti-MC K$^+$ serum; and rabbit 05-75 anti-PfEMP1 (and PfEMP3) antiserum. The prebleed and post-rC1-2 immunization bleed from rabbit 1 were analyzed in parallel.

(B and C) The anti-PfEMP1 antibodies in anti-rC1-2 (rat number 2) and anti-rD1 (rabbit) antisera immunoprecipitated PfEMP1 but do not cross-react. Antisera were preadsorbed with glutathione–Sepharose beads (none) or with GST or GST fusion proteins derived from *MCvar-1* and *MCvar-2* PfEMP1 (rB1, rC1-2, rD1) or from other P. falciparum genes (rA62-5, rPfEMP3) and used for immunoprecipitation. Only a portion of the autoradiograph is shown.

with DNA from K$^+$C$^+$ (for knob- and cytoadherence-positive) and K$^-$C$^-$ MC strain parasites, together with DNA from six other parasites of widely dispersed geographic origin and diverse adherence phenotypes (Figure 2). The A1 fragment from the 5′ end of the gene (−244–518) hybridized to DNA from all eight parasites, yielding 5–8 hybridization-positive fragments of similar size. Similar results were obtained with three other fragments from bp 3226–6446 (fragments E1, and two fragments from F-gDNA). In contrast, fragments derived from bp 455–3768, including the C1 fragment (Figure 2), and the 6692–8227 region did not hybridize with DNA from all parasites, reacting almost exclusively with MC parasites. Hence, the novel gene shares extreme 5′ sequence and the region 3644–6446 with gDNA of diverse parasites, while the central region (nucleotides 455–3768) and the 3′ region (6692–8227) show a significant difference between MC and other parasites. Since there is only a single EcoRI site, within fragment F, and no HindIII site in the sequence, the presence of multiple fragments with MC K$^+$C$^+$ parasites indicates multiple forms of the nov l gene. This is consistent with th significant sequence differenc s observed in independent cDNA clones from the same parasite.

Antib di s Generated against R combinant Fusi n Pr teins Recogniz PfEMP1

To identify the gene corresponding to the novel cDNA, we immunized laboratory animals with recombinant protein corresponding to different parts of the cDNA (see Figure 1A). The properties of the antisera are illustrated in Tables 1 and 2.

The properties of the ^{125}I–protein identified by the anti-rC1-2 and anti-rD1 antisera were identical to those of ^{125}I–PfEMP1 (Figure 3). The ^{125}I–protein comigrated with ^{125}I–PfEMP1 immunoprecipitated by three critical antisera: a pool of human immune serum that agglutinates MC K$^+$ PEs; Aotus anti-MC K$^+$ serum that specifically agglutinates and immunoprecipitates ^{125}I PfEMP1 from this strain (Howard et al., 1988); and rabbit 05-75 antiserum that immunoprecipitates MC strain ^{125}I–PfEMP1 (van Schravendijk et al., 1993). The ^{125}I–protein was not immunoprecipitated from the Triton X-100 extract of ^{125}I-labeled MC PEs and was destroyed by treatment of intact PEs with trypsin, additional properties that define ^{125}I–PfEMP1 (Figure 3A).

The anti-rC1-2 and anti-rD1 antisera did not immunoprecipitate ^{125}I–PfEMP1 from SDS or Triton X-100 extracts of the nonadherent PEs of MC K$^-$ parasites known to lack surface-exposed PfEMP1 (Aley et al., 1984), or with ItG2-ICAM parasites, even though a ^{125}I–PfEMP1 was immunoprecipitated from the latter parasites by a pool of human immune sera (Baruch et al., unpublished data). Hence, the anti-rC1-2 and anti-rD1 antisera define a MC K$^+$ strain-specific epitope(s) on ^{125}I–PfEMP1, similar to sera from Aotus monkeys infected with this parasite (Howard et al., 1988). These results are consistent with the identity of the novel gene as PfEMP1.

Preadsorption of anti-rC1-2 antiserum with glutathione–Sepharose beads bearing rC1-2 completely ablated the capacity of this antiserum to immunoprecipitate ^{125}I–PfEMP1, while beads bearing rD1, rB1, rA62-5, rPfEMP3, or GST itself had no effect (Figure 3B). In contrast, preadsorption of anti-rD1 antiserum with beads bearing rC1-2 had no effect on immunoprecipitation, while in this case, preadsorption with rD1 completely ablated immunoprecipitation (Figure 3C). We conclude that elicitation of the anti-PfEMP1 antibodies by these two recombinant proteins does not reflect spurious cross-reactivity and that GST per se is not involved in the elicitation of the anti-PfEMP1 antibodies. The anti-rC1-2 and anti-rD1 antisera independently define structural similarity between each recombinant protein and two regions of the MC K$^+$ PfEMP1. Hence, the cDNA corresponds to part of the PfEMP1 gene of the MC strain of P. falciparum.

Rabbit antisera against rC1-2 were tested for reactivity on Western blotting with proteins in SDS extracts of different P. falciparum strains and clones (Figure 4). This panel of parasites included examples with known differences in the size and antigenic specificity of their ^{125}I-labeled PfEMP1 proteins as defined by immunoprecipitation. A high mol cular mass, size-variant antigen was identified in many of the parasite samples but was missing from uninfected human erythrocytes (NRBC, Figure 4). The erythrocyte spectrin bands wer immunoreactive with all parasites and with uninfected erythrocytes. Low level cross-r activity with histidine-rich protein 1 (HRP1) was also detected (Figure 4). Th preble d of this rabbit antis - rum was also reactiv to spectrin but did not recognize th siz -variant bands (data not shown). Several pi ces

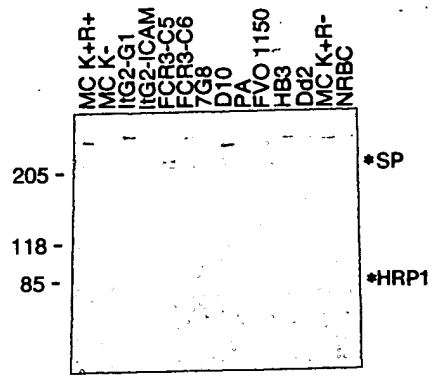

Figure 4. Immunoblotting of Diverse P. falciparum Parasites with Rabbit Anti-rC1-2 Antiserum Identifies Antigenic Cross-Reactivity among PfEMP1 Proteins

SDS extracts from 2.5 × 10^5 parasites (trophozoite and schizont stage) were subjected to SDS–PAGE, transferred to polyvinylidene difluoride membrane, and incubated with rabbit number 2 anti-rC1-2 serum for 1 hr at room temperature. Bound antibodies were visualized by the ECL Western blot method. The positions of the erythrocyte spectrin band (SP) and of HRP1 are indicated in the figure.

of evidence lead to the conclusion that the size-variant antigens (200 to >300 kDa) identified by the rabbit anti-rC1-2 antiserum are the PfEMP1 proteins of these parasites. First, the MC K^+ sample exhibited a reactive band, whereas the MC K^- sample exhibited little or no reactivity. Second, the mobility of the reactive band of FVO strain, migrating just faster than the spectrin 1.2 band, and the two to three reactive bands found in FCR_3-C5 parasites are characteristic of ^{125}I–PfEMP1 protein of these strains (van Schravendijk et al., 1991; Baruch et al., unpublished data). Third, mild trypsinization of intact MC K^+ PEs reduced the intensity of the immunoreactive band (data not shown). ItG2G1, HB3, D10, and 7G8 parasites each possessed a single reactive band but with a different apparent molecular mass, while ItG2-ICAM, Dd2, and PA parasites did not display a reactive band.

Anti-PfEMP1 Antibodies Rea t with the Surface of PEs in a Strain-Specific Manner

Each of the four rat antisera to rC1-2 react d by indirect immunofluorescence with th surfac of MC PEs and specifically agglutinated PEs of this strain (Figures 5A–5C; see Tables 1 and 2). The immunofluorescence reactivity gave a speckled pattern over the entire PE surface. Fluorescence staining was observed on ~80% of late trophozoite and schizont PEs. Preimmune rat sera or rat antisera to PfEMP3 (van Schravendijk et al., 1993) were not reactive. Uninfected erythrocytes and erythrocytes inf cted with young trophozoites or ring stages were not labeled. No reactivity was found with MC K^-, ItG2-ICAM, ItG2-G1, or FCR_3–C5 PEs.

Agglutinates of several to hundreds of intact, mature PEs were formed after incubation of infected blood with rat anti-rC1-2 antisera (Figure 5C). These agglutinates closely resembled those seen with Aotus anti-MC serum. The extent of agglutination and titer increased after subsequ nt immunizations, with titers of 1:10 to >1:100 (Table 1). Uninfected erythrocytes were not present in these agglutinates, nor were preimmune sera reactive. MC K^- PEs were not agglutinated, nor were PEs from another parasite, ItG2-ICAM, known to express an antigenically different form of PfEMP1, although these PEs were agglutinated by pooled human immune sera (Table 1). Failure of ItG2-ICAM and MC K^- PEs to be agglutinated by the anti-rC1-2 antisera correlates with the failure of such antisera to immunoprecipitate ^{125}I–PfEMP1 from these parasites.

Immunoelectron microscopy was performed to localize the reactivity of the anti-rC1-2 antibodies on the PE surface. Treatment of intact MC K^+ PEs with rat anti-rC1-2 antibodies followed by gold-conjugated goat anti-rat immunoglobulin G (IgG) yielded deposition of gold particles on the PE outer membrane (Figure 5D). Up to 30% of schizont-stage PEs were positive. The deposition of gold particles was confined to knobs (Figure 5D), with 50%–70% of the knobs labeled. Gold particles were not deposited after treatment with control antisera or rat anti-PfEMP3 antiserum. No binding was detected with ItG2-ICAM, MC K^- PEs, or uninfected erythrocytes. PfEMP1, as defin d

Table 1. Agglutination of PEs with Sera Containing Anti-PfEMP1 Antibodies

	Parasite Strain					
	MC K^+		ItG-ICAM		MC K^-	
Serum	Agglutination[a]	Titer[b]	Agglutination[a]	Titer[b]	Agglutination[a]	Titer[b]
Human immune pool	4+	125	4+	125	0	–
Aotus anti-MC K^+ (9050)	4+	125	0	–	0	–
Aotus anti-MC K^- (7925)	0	–	ND	ND	0	–
Rat 1 anti-rC1-2	4+	>100	0[c]	–	0	–
Rat 2 anti-rC1-2	3+	20	0	–	0	–
Rat 3 anti-rC1-2	1+	10	0	–	0	–
Rat 4 anti-rC1-2	3+	20	0	–	0	–
Rats 1–4 prebleeds	0	–	ND	ND	ND	ND

[a] Semiquantitative agglutination score at 1:5 serum dilution: 0, no agglutination; 1+, 10 or more agglutinates of <20 PEs; 2+, 10 or more agglutinates of 20–50 PEs; 3+, 10 or more agglutinates of 100-200 PEs; 4+, 10 or more agglutinates of >200 PEs.

[b] Reciprocal of maximum serum dilution at which PE agglutination was observed.

[c] Few, very small agglutinates (fewer than 1+) were detected at a dilution of 1:5.

ND, not determined.

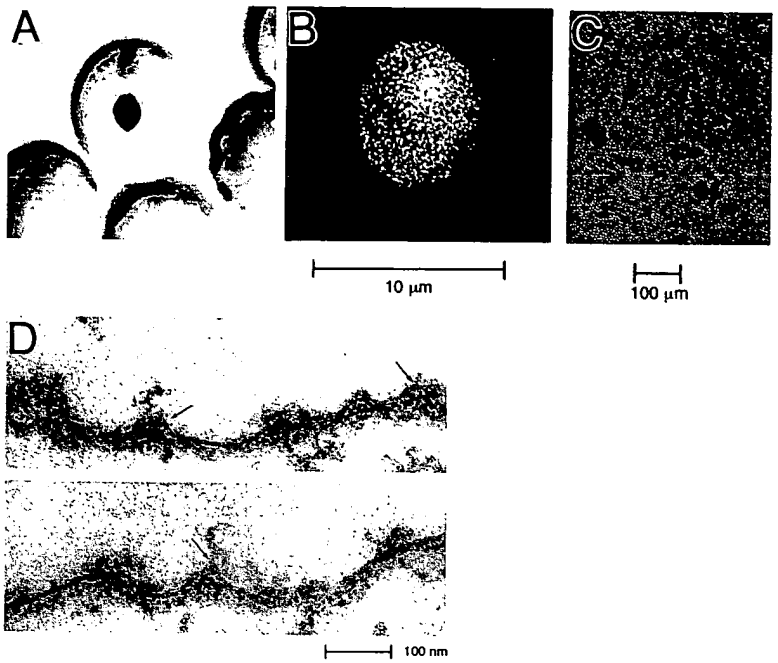

Figure 5. Antisera Raised against the rC1-2 Fragment of MC K+ PfEMP1 React with the Surface of MC K+ PEs in a Strain-Specific Manner

Results shown for anti-rC1-2 rat antiserum number 1.

(A and B) Indirect immunofluorescence of intact nonfixed PEs of MC K+, strain detected by confocal fluorescence imaging microscopy. Cells (4% parasitemia) were incubated with anti-rC1-2 antiserum and visualized by TRITC-conjugated goat anti-rat IgG.

(A) Bright field showing a pigmented (mature) infected erythrocyte and several uninfected erythrocytes.

(B) Fluorescence of the same field with reactivity only on the surface of the infected erythrocyte. The focal concentration of fluorescence is attributed to the narrow plane of confocal microscopy.

(C) Antibody-mediated PE agglutination observed by light microscopy. Anti rC1-2 antiserum (1:20 dilution) agglutinated mature MC K+ PEs. The infected blood was 8% parasitemia. Similar results obtained with other anti-rC1-2 antisera are summarized in Table 1.

(D) Immunoelectron microscopy of intact MC K+ PEs with anti-rC1-2 antiserum identified PfEMP1 expression specifically at knob protrusions. Treatment with rat antiserum was followed by treatment with 5 nm gold–conjugated goat anti-rat IgG. Gold particles were deposited on >50% of the knobs.

Scale bar in (A) and (B), 10 μm; in (C), 100 μm; in (D), 100 nm.

by the anti-rC1-2 antisera, is therefore localized on the surface membrane of PEs at knob protrusions.

Anti-PfEMP1 Antibodies Block Adherence of PEs to CD36 but Not to TSP

To test antiserum-dependent inhibition of PE adherence, PEs were preincubated with test antiserum before adding the mixture to plastic dishes coated with CD36 or TSP. Each of the four rat antisera raised against rC1-2 blocked adherence of MC K+ PEs to CD36 but had no effect on adherence to TSP (Figure 6A). In some experiments, the preimmune rat sera had an inhibitory effect of 10%–30% at 1:5 dilution, with <10% inhibition at dilutions of 1:10 or greater (Figure 6B). The inhibitory effect of preimmune sera was eliminated or markedly reduced by dialysis. This had no effect on inhibition mediated by immune rat antisera (data not shown).

Each of the rat antisera raised against rC1-2 inhibited adherence to CD36 in a dose-dependent manner. The results for the most potent rat antiserum (serum number 1; Figure 6B) show blockade of adherence titratable to 1:100 dilution with significant inhibition over the preimmune control. At 1:10 dilution, dialyzed antisera from three rats inhibited 27%–64%, whil dialyz d preimmune sera or rat anti-PfEMP3 antis rum was without effect. By comparison, Aotus anti–MC K+ sera inhibited 15% at 1:5 dilution. Non of the rat anti-rC1-2 antisera (dilution ≥1:10) blocked adh rence of ItG2-ICAM-1, PA K−C+, or ItG2-G1 PEs to CD36 (data not shown). Thes results further demonstrate that the anti-rC1-2 antisera are specific for inter-

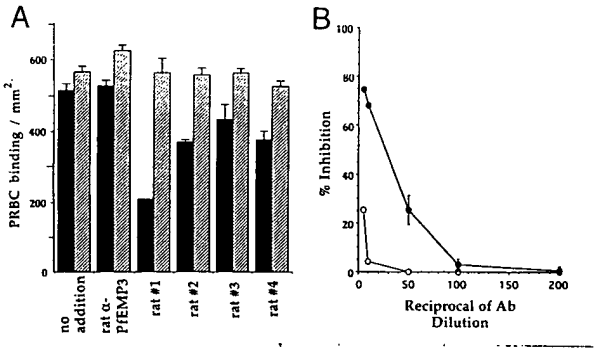

Figure 6. Antisera to the rC1-2 Fragment of MC K+ PfEMP1 Block Adherence of MC K+ PEs to Immobilized CD36 in a Dose-Dependent Manner, but Do Not Block Adherence to Immobilized TSP

PEs were preincubated with diluted antiserum before adherence to immobilized proteins was tested. The number of adherent PEs binding after washing was determined by light microscopy counting. Results shown as means and standard deviations of quadruplicate assays.

(A) PEs were preincubated with BM alone (no addition) or with BM containing 1:5 dilution of rat anti-PfEMP3 antiserum or rat anti-rC1-2 antisera (rats 1–4). Rat anti-rC1-2 antisera blocked adherence of PEs to CD36 (solid bars) but not to TSP (hatched bars). The inhibition of adherence observed with antiserum from rats 1, 2, and 4 was significantly different versus control ($p < 0.0004$).

(B) Concentration-dependent blockade of adherence to CD36 with rat anti-rC1-2. Immune (closed circles) and preimmune antiserum (open circles) from rat number 1 were tested at different dilutions for blockade of adherence of MC K+ PEs to CD36.

Table 2. Properties of Antisera against PfEMP1 Recombinant Fusion Proteins

Immunogen[a]	Species	PfEMP1 Immunoprecipitation[b]	PE agglutination[c]	PE IFA[d]	Blockade of PE Adherence[e]
rB1 (161–385)	Rabbit	0 of 2	0 of 2	ND	0 of 2
rC1-2 (576–808)	Rabbit	3 of 4	2 of 4	0 of 2	0 of 4
rC1-2 (576–808)	Rat	4 of 4	4 of 4	4 of 4	4 of 4
rD1 (818–1003)	Rabbit	2 of 2	0 of 2	ND	0 of 2

Results shown as number of antisera scoring positive for property out of number of animals immunized. ND, not defined.
[a] GST fusion proteins corresponding to fragments of MC K+ PfEMP1.
[b] Immunoprecipitation of ^{125}I–PfEMP1 from SDS extract of MC K+ PEs.
[c] Agglutination at 1:5 dilution of intact mature stage PEs of MC K+ strain.
[d] Indirect immunofluorescence with intact nonfixed MC K+ PEs.
[e] Blockade of adherence of MC K+ PEs to immobilized CD36 at 1:5 serum dilution

action with the surface of MC K+ PEs and support the idea that PfEMP1 mediates adherence of PEs to CD36.

Discussion

Properties of PfEMP1 as Previously Defined Have Been Met by the Novel Gene

PfEMP1 has been attributed the dual properties of antigenic variation on the surface of P. falciparum PEs and receptor properties of adherence to host proteins on microvascular endothelial cells (for reviews, see Howard and Gilladoga, 1989; Pasloske and Howard, 1994). PfEMP1 is therefore at the crux of understanding the molecular pathogenesis of falciparum malaria insofar as it involves antigenic variation and evasion of antimalarial immunity, and PE sequestration with consequent vascular obstruction. The molecular basis for these phenomena has languished, however, since repeated attempts to clone PfEMP1 have failed.

Several independent criteria establish that the genes described herein encode the PfEMP1 protein of K+ MC strain P. falciparum. The relevant results are summarized in Table 2.

First, PfEMP1, as identified by immunoprecipitation of ^{125}I-labeled PE surface proteins, is antigenically diverse with different parasite strains and clones (Leech et al., 1984b; Howard et al., 1988; van Schravendijk et al., 1991; Biggs et al., 1992). Antibodies that had been generated in multiple animals by immunization with recombinant proteins derived from two different parts of the cDNA immunoprecipitated ^{125}I–PfEMP1 only from MC strain PEs and failed to immunoprecipitate PfEMP1 from ItG2-ICAM PEs. The ^{125}I-immunoprecipitated protein was defined as PfEMP1 by its molecular size, specific detergent extraction properties, and sensitivity to low levels of trypsin (Aley et al., 1984; Leech et al., 1984b; Howard et al., 1988). Competition experiments with the two recombinant proteins proved that the capacity of these anti-recombinant antisera to immunoprecipitate ^{125}I–PfEMP1 was not due to the presence of cross-reactive epitopes on these immunogens. Western blotting with different strains and clones indicated that the anti-rC1-2 antiserum was reactive with bands of variable size, including some that show migration similar to that of ^{125}I–PfEMP1 from these strains (Howard t al., 1988; van Schravendijk et al., 1991; Baruch et al., unpublished data). However, much more cross-reactivity is apparent among fully unfolded PfEMP1s, as with Western blotting, than among native PfEMP1 proteins expressed on the PE surface.

Second, since PfEMP1 is expressed as a variant protein on the PE surface (Howard et al., 1988; van Schravendijk et al., 1991; Roberts et al., 1992), antisera raised against the recombinant protein should react in a strain-specific manner with the surface of intact PEs. Rat anti-rC1-2 antisera reacted with the surface of intact MC K+ PEs by mediating specific agglutination, by indirect immunofluorescence, and by immunoelectron microscopy. This reactivity was specific to the MC K+ strain and was not evident with any other strain, including the MC K− strain, known to lack surface-exposed PfEMP1 (Aley et al., 1984). Reactivity of these anti-PfEMP1 antibodies on immunoelectron microscopy was restricted to the knob protrusions on PEs, in agreement with earlier studies that demonstrated specific binding of isolate-specific antibodies to knobs (van Schravendijk et al., 1991) and specific binding of CD36 and TSP to knobs (Nakamura et al., 1992).

Finally, PfEMP1 has been associated with the property of adherence of PEs to CD36 and other endothelial cell surface proteins (Howard and Gilladoga, 1989). Antisera raised against rC1-2 specifically blocked PE adherence to CD36. The ability of antisera to block adherence of PEs was generally correlated with agglutination of the same PEs (Howard et al., 1988). The results obtained with the anti-rC1-2 antisera support and verify these observations.

Structural and Functional Aspects of the MC PfEMP1 Gene Product

Analysis of the MC PfEMP1 sequence would predict that each cDNA clone would hybridize to a single band of EcoRI or EcoRI–HindIII-digested DNA. In contrast, hybridization to more than one band was apparent with every clone, indicating more than one gene copy in the parasite genome. This is supported by the results of the accompanying papers (Smith et al., 1995; Su et al., 1995 [both in this issue of *Cell*]) and our own data presenting two variants of the MC strain PfEMP1 gene. Thus, P. falciparum parasites have a family of PfEMP1 genes that constitute shared and diverse sequences. PfEMP1 displays antigenic variation with extremely high frequency (Roberts et al., 1992). Antigenic switching is associated with expression of a different

PfEMP1 gene (Smith et al., 1995). Thus, antigenic differences in the PfEMP1 extracellular domains, a property expected from the earlier serology (Marsh and Howard, 1986; Aguiar et al., 1992) and immunochemical studies (Leech et al., 1984b; Howard et al., 1988), must derive from these sequence differences.

Antibodies to rC1-2 block PE adherence to CD36 but not to TSP. In separate studies, we have demonstrated that different tryptic fragments of ^{125}I–PfEMP1 released from the surface of MC K$^+$ PEs bind to TSP and CD36, suggesting that these receptor properties reside in different parts of the PfEMP1 protein (Baruch et al., unpublished data). The capacity of anti-rC1-2 antibodies to block adherence to CD36 but without effect on adherence to TSP is consistent with these observations. We suggest that the CD36-binding portion of PfEMP1 may reside within the region corresponding to rC1-2. However, blockade of adherence by the anti-rC1-2 antisera is strain specific, and the anti-rC1-2 antibodies may have sterically hindered the CD36-binding site or elicited alterations in the shape of PfEMP1 such that it can no longer bind to CD36. Studies are underway to explore these possibilities.

Within the protein, large domains can be identified with clear homology to the DBL domains characteristic of EBA-175 and Duffy antigen–binding protein (Su et al., 1995), which are known to participate in the binding of merozoite surface proteins to erythrocyte proteins (Chitnis and Miller, 1994). The MC sequence possesses four of these domains, defined by particular sequence motifs, as do the other PfEMP1 genes cloned to date (Su et al., 1995). The rC1-2 recombinant protein that elicited adherence-blocking antibodies lacked any portion of the DBL domains but included the CRM-1 cysteine-rich motif. Also of potential relevance to the adherence properties of PfEMP1 was the observation of an RGD motif and an LDV motif, both associated with protein–protein interaction and cell attachment (for review, see Kuhn and Eble, 1994). These motifs occur in some PfEMP1 sequences but not in others. *MCvar-1* includes an RGD motif, while *MCvar-2* lacked this motif. Additional studies are required to explore the role of these motifs in PE adherence to endothelial cells. The appearance of such motifs in only some of the PfEMP1 genes may explain the extraordinary diversity and plasticity of the PE adherence phenotype.

The central role of PfEMP1 in P. falciparum biology, as the malarial adherence receptor for host proteins on microvascular endothelium, led us to suggest its inclusion in a malaria vaccine and also as a target for novel therapeutics to reverse PE adherence in acute cerebral malaria (Howard and Gilladoga, 1989). Before part of PfEMP1 can be incorporated in a vaccine, an antigenically conserved extracellular portion of the PfEMP1 protein must be identified, insofar as it would elicit antibodies capable of blocking adherence of all P. falciparum parasite variants to endothelial cells. The independent goal of novel drugs to reverse the obstruction of cerebral microvessels caused by adherent PEs will demand that such a compound bind to PfEMP1 proteins of all parasites. These concepts can now be test d experimentally.

Experimental Procedures

Parasites

The Malayan Camp MC K$^+$R$^+$C$^+$ (knob-, rosette-, and cytoadherence-positive) line of P. falciparum, denoted MC K$^+$, and the ItG2-ICAM K$^+$C$^+$ clone (Ockenhouse et al., 1991) were maintained in culture with O$^+$ erythrocytes (Handunnetti et al., 1992). DNA or proteins were also extracted from the following P. falciparum parasites: MC K$^+$C$^+$R$^-$; MC K$^-$C$^-$; ItG2-G1 K$^+$C$^+$; FCR$_3$–C5 K$^+$C$^+$; FCR$_3$–C6 K$^-$C$^-$; 7G8 K$^+$; Palo Alto, PA K$^-$C$^+$; D10 K$^+$C$^+$ (only TSP), and Dd2 (K$^+$, low C$^+$). FVO was derived from Aotus monkey 1150. C$^+$ refers to adherence to both CD36 and TSP.

Preparation of Nucleic Acids

P. falciparum gDNA was isolated from mature PEs by lysis in NP-40 (Pasloske et al., 1993). DNA for Southern blotting was digested with EcoRI or EcoRI–HindIII and blotted under high stringency (Pasloske et al., 1993).

Screening of the MC gDNA Expression Library

The genomic DNA λgt11 expression library and screening of the library with antibodies have been described (Pasloske et al., 1993). Rabbit antiserum 05-75 (van Schravendijk et al., 1993) was exhaustively adsorbed with the λgt11–12.1.3 clone (Pasloske et al., 1993) and used at 1:150 dilution for screening the gDNA library. Clones so derived were subcloned into the SK$^-$ vector (Stratagene Cloning Systems, La Jolla, CA).

Isolation of cDNA Clones

The MC K$^+$ cDNA library was prepared in the expression plasmid pJFE14DAF as described by Elliott et al. (1990). PfEMP1 clones were isolated from the cDNA library by using a modified version of the leapfrog method (Gibbons et al., 1991). cDNA clones extending 5′ or 3′ from previously isolated cDNA and gDNA clones were generated by PCR using an oligonucleotide primer 100–150 bases from the proximal terminus region of the cloned sequence and another primer derived from the vector arm flanking the cloning site. PCR was performed on 10 ng of the cDNA library by using 30 cycles with a profile of 1 min at 94°C, 1 min at 55°C, and 2.5 min at 72°C. The PCR products were fractionated on low melt agarose gels, and products of 1–3 kb were gel purified (Wizard PCR preps, Promega, Madison, WI), cloned into pAMP vector (Life Technologies, Incorporated, Gaithersburg, MD), and transformed into E. coli DH5-α maximum efficiency cells (Life Technologies, Gaithersburg, MD) or Sure-2 cells (Stratagene Cloning Systems, La Jolla, CA). To facilitate cloning, the PCR primers were designed with 5′ adaptor sequences for directional insertion into the pAMP vector. PfEMP1 clones were identified by direct colony hybridization using end-labeled oligonucleotides designed 50–100 bases internal to the sequence-specific PCR primer. The F-gDNA clones were generated by PCR cloning from the MC parasite gDNA as described by Su et al. (1995).

DNA Sequencing and Analysis

Plasmid DNA was isolated from the recombinant clones by using the Wizard Miniprep DNA isolation system (Promega, Madison, WI), alkali denatured, and sequenced via the dideoxy chain termination method using the Sequenase kit (United States Biochemical Corporation, Cleveland, OH). Either vector-specific or custom oligonucleotide primers were used for primer-directed sequencing. For some of the clones, the insert was subcloned into the pBluescript SK$^-$ vector (Stratagene Cloning Systems, La Jolla, CA) and unidirectional deletion mutants generated by using exonuclease III (Henikoff, 1984). To ensure that new clones were continuous with the initial ones, primers from within the two clones were used to generate PCR products from MC gDNA and the cDNA library, and the identity of the generated product was confirmed by size, hybridization with oligonucleotides, and direct sequencing (fmol sequencing system, Promega, Madison, WI). Sequences were analyzed with DNASTAR (DNASTAR Incorporated, Madison, WI) sequence analysis software.

Glutathione S-Transferase Fusion Proteins

Glutathione S-transferase (GST) fusion proteins were constructed by

cloning of PCR products carrying a BamHI site at the 5′ end and an EcoRI site at the 3′ end into the BamHI–EcoRI sites of the pGEX-3X vector (Amrad Corporation, Limited). The recombinant fusion proteins generated were as follows: rB1, amino acids 161–385; rC1-1, amino acids 402–605; rC1-2, amino acids 576–808; and rD1, amino acids 818–1003. The GST fusion proteins were expressed in E. coli DH5-α or Sure-2 cells and purified on glutathione–Sepharose (Pharmacia LKB Biotechnology, Piscataway, NJ) (van Schravendijk et al., 1993), except that PBS was replaced with a column buffer (20 mM Tris, 200 mM NaCl [pH 7.5]). rC1-1 did not bind to the glutathione resin and could not be purified. rA62-5 was derived from clone A62, and rPfEMP3 corresponded to the 12.1.3 recombinant protein (van Schravendijk et al., 1993).

Antibodies

Mouse MAb 179 recognizes an epitope sequence incorporated into the carboxyl terminus of sCD36 expressed as a phosphoinositol glycan–linked extracellular domain (Affymax Research Institute). Rabbit antiserum 05-75, which recognizes both PfEMP3 and MC PfEMP1, was described previously (van Schravendijk et al., 1993). A human immune serum pool was prepared from five individuals resident in a P. falciparum–endemic area of Ghana (van Schravendijk et al., 1993). Aotus anti–P. falciparum sera 779 and 9050 were derived from animals infected with the Aotus MC K^+ strain and drug cured (Leech et al., 1984b).

Recombinant proteins (rB1, rC1-2, and rD1) bound to glutathione–Sepharose 4B beads (Pharmacia) were used to immunize rabbits (0.1 mg of recombinant protein) and rats (0.05 mg of recombinant protein). Initial immunization was performed with Freund's complete adjuvant followed by booster immunizations with Freund's incomplete adjuvant at days 21, 35, 49, and 63, and then monthly. Animals were bled 7 days after each boost.

Surface Iodination, Trypsinization, Sequential Extraction, and Immunoprecipitation

Mature intact PEs were enriched to >90% by the Percoll–Sorbitol method (Kutner et al., 1985) after initial disruption of rosettes (Handunnetti et al., 1992), iodinated by the lactoperoxidase method, and sequentially extracted with 1% (w/v) Triton X-100 followed by 2% (w/v) SDS (van Schravendijk et al., 1993). For trypsinization, iodinated PEs were incubated at 10% hematocrit with 10 μg/ml of trypsin–TPCK (Sigma, St. Louis, MO) in PBS for 5–10 min at 21°C, and trypsinization was terminated as described (Leech et al., 1984b). ^{125}I–SDS extracts (5–7 μl) or ^{125}I–Triton X-100 extracts (10–15 μl) were immunoprecipitated as described (van Schravendijk et al., 1993). For depletion of anti-PfEMP1 antibodies with PfEMP1-recombinant proteins, 7.5 μl of antisera was incubated 3 hr at 21°C in 500 μl of NETT–BSA containing 15 μg of recombinant protein bound to glutathione–Sepharose 4B beads. The beads were centrifuged and removed, 5 μl of ^{125}I–SDS extract added, and immunoprecipitation performed.

Western Blots

Trophozoite-stage PEs were extracted sequentially with 1% Triton X-100 and 2% SDS to a final concentration of 10^8 parasites per milliliter. Extracts (2.5 μl) were SDS–PAGE-fractionated on 5% polyacrylamide gels, transferred onto Immobilon P membrane (Millipore Corporation, Bedford, MA), and immunoblotted by using an enhanced chemiluminescence (ECL) Western blotting protocol (Amersham International, Buckinghamshire, England). Membranes were incubated overnight at 5°C in 50 mM Tris, 150 mM NaCl, 0.1% Tween 20 (pH 8.0) (TBS-T) containing 10% (w/v) nonfat dry milk, followed by a 1 hr incubation with the primary antibody diluted in TBS-T, 5% (w/v) dry milk (TTM), one wash with TBS-T, two with high-salt TTM (0.5 M NaCl), and two with TTM. Horseradish peroxidase–conjugated donkey anti-rabbit IgG (Jackson Immunoresearch Laboratories, Incorporated) was diluted 1:50,000 in TTM and added to the membranes for 45 min. The membranes were washed once with TBS-T, twice with high-salt TTM, three times with TTM containing 0.5% Triton X-100, and three times with TBS-T.

PE Agglutination

Agglutination assays were performed as described earlier (Aguiar et al., 1992). PEs were washed twice with RPMI 1640 (Roswell Park Memorial Institute), 25 mM HEPES, 1% BSA (pH 6.8 or 7.2) (binding media [BM]) and resuspended to 20% hematocrit. Blood (15 μl) was mixed with an equal volume of diluted antisera, incubated 45 min at 37°C in a G24 environmental incubator shaker (New Brunswick Scientific) with continuous rotation (125 rpm), examined microscopically, and scored on the basis of the size and number of the PE agglutinates.

Soluble Receptors and Cytoadherence Microassay

Soluble CD36 was obtained in the form of harvest supernatant (approximately 1–2 μg/ml) by cleaving phosphoinositol glycan–linked CD36 from the surface of stable transfected CHO cells with phosphatidylinositol-specific phospholipase C (Lin et al., 1990) and stored at 5°C. Purified TSP was purchased from GIBCO BRL. A modification of a microscopic adherence microassay (Hasler et al., 1993) was used for antibody-mediated inhibition of PE adherence. MAb 179 (7 μl at a concentration of 50 μg/ml in PBS) was used to coat each well (1 hr at 21°C), washed once with 50 μl of PBS, blocked 30 min at 21°C with PBS containing 1% BSA, and washed twice with PBS. sCD36 (50 μl, usually 0.2, 0.4, or 2 μg/ml) was added and incubated 1 hr at 21°C. TSP (50 μg/ml) was coated directly on the plastic (2 hr at 21°C). Each well was washed twice with BM. PEs were washed once with BM and resuspended to 4% hematocrit in BM plus 10% FCS. An equal volume of diluted antiserum was added and the cells incubated with the antisera for 1 hr at 37°C. Cells (50 μl) at 2% hematocrit were added to each well, incubated 1 hr at 37°C, washed four times with BM, and then fixed, stained, and counted (Hasler et al., 1993).

Confocal Fluorescence Imaging Microscopy

Immunofluorescence microscopy was performed with a Bio-Rad MRC-600 system (Bio-Rad Laboratories, Cambridge, MA) interfaced to an Olympus IMT-2 inverted microscope as described previously (Gormley et al., 1992). Cells (1 × 10^6; 4% trophozoite and schizont stages) were washed three times in RPMI 1640, resuspended in 10 μl of antiserum, and incubated 2 hr at 37°C with constant shaking. The samples were washed three times with RPMI and rhodamine–TRITC-labeled goat anti-rat IgG (Jackson Immunoresearch Laboratories, Incorporated) added at a 1:10 dilution in RPMI for 30 min at 37°C. The cells were washed three times in RPMI, diluted to 0.3% hematocrit, and viewed in a Dvorak chamber.

Immunoelectron Microscopy

Cells (5% hematocrit, 5% parasitemia) were incubated in RPMI containing 2% BSA for 30 min at 25°C with constant shaking. Primary rat antiserum was added at 1:100 dilution for 1 hr at 25°C with constant shaking, followed by three washes with RPMI. We then incubated 5 nm gold–conjugated goat anti-rat IgG (Goldmark Biologicals) with the cells at a 1:50 dilution in RPMI for 30 min at 25°C. The cells were washed three times with RPMI, fixed overnight at 4°C in 2% glutaraldehyde, 1% tannic acid, 4% sucrose, 0.1 M phosphate buffer (pH 7.4), washed with 0.1 M phosphate buffer, and postfixed in 2% osmium tetroxide in 0.05 M phosphate buffer (pH 7.4) on ice for 90 min. The specimens were washed four times with deionized water and incubated with 1% uranyl acetate for 15 min at 25°C, washed four times with deionized water, embedded in 2% agarose, and dehydrated in graded steps of acetone. The cells were infiltrated and embedded in Spurr's. Thin sections were cut on a Reichert–Jung Ultracut E ultramicrotome and poststained with 2% uranyl acetate and 1% lead citrate. The sections were examined with a Hitachi H-7000 scanning transmission electron microscope.

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GenBank Accession Numbers

The accession numbers for the sequences reported in this paper are U27338 (*MCvar-1*) and U27339 (*MCvar-2*).